Flavonoids Are Involved in Regulating Salt Stress Tolerance in Atriplex centralasiatica Seedlings (Postprint)

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Abstract

Atriplex centralasiatica is a typical halophyte widely distributed in saline-alkali regions of China. In previous studies, we found that salt stress significantly induced flavonoid accumulation in the root tips of Atriplex centralasiatica seedlings, but the underlying physiological mechanism remains incompletely understood. Under treatments with different salt concentrations (50 $\mathrm{mmol \cdot L^{-1}}$, 100 $\mathrm{mmol \cdot L^{-1}}$, 250 $\mathrm{mmol \cdot L^{-1}}$, 500 $\mathrm{mmol \cdot L^{-1}}$, 600 $\mathrm{mmol \cdot L^{-1}}$ NaCl), through the addition of KNO_3 (25 $\mathrm{mmol \cdot L^{-1}}$, 35 $\mathrm{mmol \cdot L^{-1}}$) and exogenous quercetin (100 $\mathrm{nmol \cdot L^{-1}}$ quercetin), and employing research methods in plant physiology and biochemistry, we analyzed the physiological mechanism of salt-induced flavonoid accumulation in regulating salt tolerance of Atriplex centralasiatica seedlings. The results showed that treatment with the exogenous flavonoid quercetin, on the one hand, further inhibited primary root growth of seedlings under salt stress, while also significantly reducing H_2O_2 accumulation in roots and MDA levels in seedlings under salt stress, but significantly increasing soluble sugar content. These results indicate that quercetin affects the root architecture of Atriplex centralasiatica by inhibiting primary root growth; and improves the salt stress tolerance of Atriplex centralasiatica seedlings by reducing oxidative damage. Salt stress also significantly induced nitrate (NO_3^-) accumulation in Atriplex centralasiatica seedlings, and treatment with 25 $\mathrm{mmol \cdot L^{-1}}$ and 35 $\mathrm{mmol \cdot L^{-1}}$ KNO_3 significantly induced flavonoid accumulation in seedling root tips. These results suggest that nitrate nitrogen may participate in regulating the salt tolerance of Atriplex centralasiatica seedlings by influencing the flavonoid pathway. This study provides a theoretical foundation for further elucidating the physiological and molecular mechanisms of salt tolerance in Atriplex centralasiatica, as well as the physiological and molecular mechanisms by which flavonoids participate in regulating salt tolerance in Atriplex centralasiatica; and simultaneously provides theoretical basis and technical support for improving crop salt tolerance and ameliorating saline-alkali soils using modern biotechnology and agronomic measures.

Full Text

Preamble

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Flavonoid is associated with salt stress tolerance in *Atriplex centralasiatica* seedlings*

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Abstract

Atriplex centralasiatica is a typical halophyte that widely grows in saline areas of China. In earlier studies, we found that salt stress markedly induced the accumulation of flavonoid in the roots of *A. centralasiatica* seedlings. By integrating physiological and biochemical analyses, this study investigated the physiological mechanisms of salt-induced flavonoid accumulation in the roots of *A. centralasiatica* seedlings by supplementing with KNO_3 (25 and 35 $mmol \cdot L^{-1}$) and exogenous quercetin (100 $nmol \cdot L^{-1}$) under different salinity treatments (50, 100, 250, 500, and 600 $mmol \cdot L^{-1}$ NaCl). Our results indicated that supplementation of exogenous quercetin markedly suppressed primary root (PR) growth and the accumulation of both H_2O_2 and MDA in roots, whereas it markedly increased soluble sugar levels in seedlings. This indicated that exogenous flavonoid—quercetin—affected root system architecture by suppressing PR growth and improved salt tolerance by reducing oxidative damage in *A. centralasiatica* seedlings. Salt stress also induced the accumulation of nitrate (NO_3^-) in *A. centralasiatica* seedlings. We found that treatment with 25 and 35 $\text{mmol} \cdot \text{L}^{-1}$ KNO_3 markedly induced the accumulation of flavonoid in root tips of *A. centralasiatica* seedlings. All in all, the results indicated that nitrate modulated salt tolerance in *A. centralasiatica* seedlings via the flavonoid pathway. This knowledge was helpful for further elucidating the physiological and molecular mechanisms of salt tolerance in *A. centralasiatica*. It also provided further insights into novel strategies for breeding and cultivating salt-tolerant crops and improving saline-alkali soils using modern biotechnology and agricultural measures.

Keywords: *Atriplex centralasiatica*; Salt stress; Flavonoid; Nitrate; Roots

Introduction

Soil salinization is a major constraint causing poor crop growth and reduced quality and yield. Research has shown that salt toxicity in plants includes osmotic stress, ion toxicity, oxidative stress, and nutritional imbalance [1-2]. First, high external salt ion concentrations decrease water potential, making water uptake difficult and causing osmotic stress [3]. Salt stress reduces the activity of antioxidant enzymes, promotes reactive oxygen species (ROS) accumulation, and induces membrane lipid peroxidation [4]. From a plant nutrition perspective, salt stress inhibits the absorption and accumulation of nutrient elements (such as N, P, Zn, Fe, etc.) by roots, thereby affecting growth [5].

Atriplex centralasiatica is a typical halophyte widely distributed in saline areas of North and Northwest China. Both pot and field experiments have shown that the average salt tolerance capacity of *A. centralasiatica* is 15 $\text{g}(\text{Na}^+) \cdot \text{kg}^{-1}(\text{soil})$, with a salt tolerance limit of 20 $\text{g}(\text{Na}^+) \cdot \text{kg}^{-1}(\text{soil})$ [6]. In recent years, the salt tolerance mechanisms of *A. centralasiatica* have been studied in greater depth [6-7].

Flavonoids are important secondary metabolites in plants that are extensively involved in regulating various biological processes related to plant growth, development, and stress resistance. Flavonoid compounds such as naringenin and rutin extracted from apple (*Malus pumila*) peel can effectively prevent DNA damage [8]. Plants with lower flavonoid content exhibit higher sensitivity to UV radiation [9-10]. Many studies have shown that flavonoids participate in regulating polar auxin transport in plants [11-12]. *Arabidopsis thaliana* transparent testa (tt) mutants defective in flavonoid synthesis show increased auxin transport capacity and consequent phenotypic changes [13]. These studies demonstrate that flavonoids play important regulatory roles in plant growth and development.

Plant nitrogen nutrition mainly includes ammonium nitrogen (NH_4^+) and nitrate nitrogen (NO_3^-). However, massive absorption of ammonium nitrogen by plants can lead to ammonium toxicity, whereas nitrate nitrogen absorption does not cause harm [14]. Therefore, as external nitrogen supply increases, plants can typically absorb nitrate in excess of their growth requirements, with a portion being reduced to NH_4^+ by nitrate reductase (NR) and the remainder stored in vacuoles [15]. This stored NO_3^- in vacuoles helps maintain cellular osmotic balance [16]. Song et al. [17] reported that in *Suaeda salsa*, a euhalophyte, NO_3^- accumulation capacity was positively correlated with salt tolerance. In barley (*Hordeum vulgare*), salt-sensitive varieties showed significantly decreased NO_3^- accumulation under salt stress, whereas salt-tolerant varieties exhibited increased NO_3^- accumulation [18]. Studies on the halophyte *Suaeda physophora* have shown that under long-term salt stress, a favorable NO_3^- transport protein system forms [19]. However, whether NO_3^- merely provides osmotic adjustment substances or participates in specific salt tolerance mechanisms remains unclear. In previous research, we found that salt stress significantly induced flavonoid accumulation in the root tips of *A. centralasiatica* seedlings, but the underlying physiological mechanisms remain unclear. Therefore, this study used *A. centralasiatica* as material to investigate the role of flavonoids in regulating salt stress tolerance in seedlings. These results provide a theoretical foundation for further elucidating the physiological and molecular mechanisms of flavonoid involvement in plant salt tolerance and offer theoretical basis and technical support for improving crop salt tolerance and reclaiming saline-alkali lands using modern biotechnology and agronomic measures.

Materials and Methods

1.1 Plant Materials and Experimental Design

Atriplex centralasiatica seeds were provided by the Nanpi Eco-Agricultural Experimental Station, Chinese Academy of Sciences. Yellow seeds of *A. centralasiatica* were used as experimental material. All plant materials were grown hydroponically in a phytotron under the following conditions: light intensity 300 mol $\cdot$ m^{-2} $\cdot$ s^{-1}, photoperiod 14 h, temperature (28$±1)°*C.Seedsweregerminatedonmoistdouble−layerfilterpaper.TheHoaglandnutrientsolutionformulatio*
*day−oldseedlingsweretransferredtovarioustreatmentsolutions.Salttreatmentconcentrationswere*0, 50, 100,
L^{-1}$ NaCl; nitrate treatments used 25 and 35 mmol $\cdot$ L^{-1} KNO_3; exogenous quercetin treatment concentration was 100 nmol $\cdot$ L^{-1}. Seedlings were cultured at 25°C, 3,000 lx, with a 16 h/8 h photoperiod for 2 days before sampling for physiological analysis. Each treatment was replicated three times.

1.2 Determination of Plant Physiological Indices

1) **Root length measurement**: After 2 days in treatment solutions, roots were spread flat in petri dishes and scanned using an EPSON PERFEC-

TION V350 PHOTO scanner. Root length was measured using Photoshop software. Thirty seedlings per treatment were used for statistical analysis.

2) **Flavonoid fluorescence detection**: Following the method of Murphy et al. [13], DPBA (2-aminoethyl diphenylborinate) fluorescent dye was used to analyze flavonoid content in root tips. After 2 days in treatment solutions, 2 cm root tips were excised and stained in DPBA working solution. Fifteen primary root tips per treatment were analyzed.
3) **Soluble sugar, H_2O_2, NO_3^-, and MDA content determination**: After 2 days in treatment solutions, roots or whole seedlings were excised and 500 mg fresh weight was accurately weighed and placed in EP tubes for biochemical analysis. Soluble sugar, NO_3^-, H_2O_2, and MDA contents were determined following the methods of Xu et al. [7,20]. Whole seedlings were used for soluble sugar and MDA content determination, while roots were used for NO_3^- and H_2O_2 content determination. Three replicates were performed.

Results

2.1 Salt Stress Induced Flavonoid Accumulation in *A. centralasiatica* Root Tips

To determine whether salt stress affects flavonoid levels in *A. centralasiatica* root tips, DPBA fluorescent dye was used to detect flavonoid content under salt stress. As shown in [Figure 1: see original paper], DPBA fluorescence staining analysis indicated that salt stress significantly induced flavonoid accumulation in *A. centralasiatica* seedling roots, and fluorescence intensity gradually increased with rising salt concentrations. However, at high salt concentrations (500 and 600 $\text{mmol}\cdot\text{L}^{-1}$ NaCl), DPBA fluorescence decreased significantly.

2.2 Exogenous Quercetin Inhibited Primary Root Growth in *A. centralasiatica* Seedlings Under Salt Stress

To clarify the role of flavonoids in regulating root growth of *A. centralasiatica* seedlings under salt stress, quercetin was applied to analyze the effects of exogenous flavonoids on seed germination and early seedling root length under salt stress. Quercetin is an important flavonoid widely used to study flavonoid functions in plant growth and development [10]. As shown in [Figure 2: see original paper], 100 $\text{nmol}\cdot\text{L}^{-1}$ quercetin treatment significantly inhibited primary root growth in *A. centralasiatica* seedlings under salt stress. Salt stress alone significantly inhibited primary root growth, and exogenous quercetin further suppressed primary root elongation. These results suggest that salt stress-induced quercetin accumulation in root tips may be one reason for primary root inhibition.

2.3 Exogenous Quercetin Increased Soluble Sugar Content and Decreased MDA Content and Root H_2O_2 Levels Under Salt Stress

To further understand the effects of flavonoids on salt tolerance in *A. centralasiatica* seedlings, we analyzed the impacts of quercetin on soluble sugar, MDA, and root H_2O_2 levels under salt stress. Salt stress significantly reduced leaf soluble sugar content. Under salt stress, seedlings treated with exogenous quercetin showed significantly higher leaf soluble sugar content compared to seedlings subjected to salt stress alone ([Figure 3: see original paper]A). Salt stress significantly increased leaf MDA content and root H_2O_2 levels. Under salt stress, quercetin-treated seedlings exhibited significantly lower leaf MDA content compared to salt-stressed seedlings ([Figure 3: see original paper]B). Quercetin treatment also significantly reduced root H_2O_2 levels ([Figure 3: see original paper]C). These results indicate that while exogenous quercetin treatment inhibited primary root growth, it enhanced seedling salt tolerance.

2.4 Salt Stress Induced NO_3^- Accumulation in Roots of *A. centralasiatica* Seedlings

Previous studies have shown that plant NO_3^- accumulation capacity is positively correlated with salt tolerance [17-18]. Therefore, we examined NO_3^- accumulation levels in *A. centralasiatica* seedling roots under salt stress. We found that salt stress significantly affected NO_3^- levels in seedling roots. Under 0–250 $\text{mmol} \cdot \text{L}^{-1}$ salt stress, root NO_3^- content gradually increased; however, at higher salt concentrations (500 and 600 $\text{mmol} \cdot \text{L}^{-1}$ NaCl), root NO_3^- content decreased significantly ([Figure 4: see original paper]).

2.5 NO_3^- Treatment Induced Flavonoid Accumulation in Root Tips

To determine whether NO_3^- accumulation in seedlings affects flavonoid levels, DPBA fluorescence staining was used to analyze flavonoid levels in root tips under different NO_3^- treatments. As shown in [Figure 5: see original paper], DPBA fluorescence in *A. centralasiatica* seedling root tips was significantly enhanced under different KNO_3 concentrations. These results indicate that NO_3^- accumulation in seedlings increased root tip flavonoid levels.

Discussion

Typically, salt stress inhibits plant NO_3^- uptake and transport, thereby exacerbating osmotic stress. For example, NO_3^- absorption and transport in *Arabidopsis thaliana* and wheat (*Triticum aestivum*) seedlings are inhibited by high salt concentrations. However, halophytes show distinct differences. Some studies have demonstrated that halophytes such as *Suaeda salsa* exhibit a positive correlation between NO_3^- accumulation capacity and salt tolerance [17-18]. Research on barley also indicated that salt-tolerant varieties showed enhanced NO_3^- uptake, whereas salt-sensitive varieties exhibited significantly decreased NO_3^- accumulation under salt stress [17-18]. These studies suggest that NO_3^-

absorption and accumulation are important physiological mechanisms for halophytes to maintain salt tolerance, though the underlying mechanisms remain unclear. This study found that different salt concentrations significantly induced NO_3^- accumulation in both roots and leaves of *A. centralasiatica* seedlings, indicating that NO_3^- may participate in regulating the salt stress response in *A. centralasiatica*. Further research revealed that both salt stress and NO_3^- treatment significantly enhanced flavonoid accumulation in *A. centralasiatica* seedling roots. Seedlings treated with exogenous flavonoids showed inhibited primary root growth but increased leaf chlorophyll and soluble sugar contents, while MDA content and H_2O_2 levels were reduced. These results suggest that flavonoids altered root system architecture and enhanced antioxidant capacity in *A. centralasiatica* seedlings, thereby improving salt tolerance.

Primary root growth is regulated by both ROS signaling pathways and auxin-cytokinin pathways [21]. High H_2O_2 concentrations in root tips inhibit primary root growth, whereas O_2^- accumulation in the root meristematic zone promotes primary root elongation [21-22]. Therefore, plants precisely regulate ROS balance in root tips to determine primary root growth [21]. Another important signaling pathway affecting primary root growth is the auxin-cytokinin pathway. Cytokinin influences the gradient distribution of PLETHORA (PLT) proteins in root tips by affecting Aux/IAA proteins, thereby regulating root meristem development and primary root growth [23]. Flavonoids are important secondary metabolites with antioxidant and ROS scavenging capacities [24-25] and also affect normal IAA metabolism and interfere with auxin transport [25-27]. In this study, exogenous quercetin treatment reduced H_2O_2 content in *A. centralasiatica* seedling root tips but inhibited primary root growth, indicating that the inhibitory effect of exogenous flavonoids on primary root growth was not mediated through ROS signaling pathways. Previous studies have shown that flavonoids are inhibitors of auxin transport [11-12]. Exogenous quercetin treatment may inhibit the accumulation of PIN auxin efflux proteins in root tips, reducing polar auxin transport from shoots to root tips and decreasing auxin accumulation, thereby affecting primary root growth [25-26]. We hypothesize that exogenous quercetin inhibits primary root growth in *A. centralasiatica* seedlings through this pathway; however, the detailed molecular mechanisms require further investigation. Our results demonstrate that flavonoids accumulated in *A. centralasiatica* seedling roots under salt stress participate in regulating salt-induced root architecture changes.

In conclusion, this study shows that salt stress induced NO_3^- accumulation in *A. centralasiatica* seedlings, which subsequently increased flavonoid levels. Flavonoids alleviated salt-induced oxidative damage by reducing root MDA content and H_2O_2 levels, thereby enhancing seedling salt tolerance. Meanwhile, salt stress-induced flavonoid accumulation also inhibited primary root growth, altering root system architecture. These findings indicate that salt stress-induced NO_3^- accumulation in *A. centralasiatica* seedlings may participate in regulating salt tolerance through the flavonoid pathway. This study reveals a novel function of flavonoids in regulating plant abiotic stress tolerance and provides a theoretical basis for further understanding flavonoid biological functions and improving plant salt tolerance through biotechnology.

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